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13. ABSTRACT *(Maximum 200 words)*

Previously, our laboratory demonstrated that c-Jun N-terminal kinase participates in apoptosis signaling. JNK induction is differentially regulated by mitogenic and apoptotic stimuli in T cells, and the duration of JNK activation may determine cell fate in T cells. Here, we show that JNK is also differentially regulated by mitogenic and apoptotic stimuli in breast cancer cell line MCF-7, suggesting that duration of JNK activation may also determine cell fate in breast cancer cells. Tumor suppressor p53 is not required for radiation induced JNK activation. It is also not required for apoptosis induced by JNK activation. However, our data does not exclude the possibility that p53 may mediate JNK-induced apoptosis, which needs to be further studied. Both JNK activity and Fas expression can be induced by γ radiation; however, Fas expression is closely associated with a wild-type p53 status but not with the JNK activation. These results suggest that Fas is not the downstream target for the JNK pathway. We demonstrate that JNK is activated through oxidative stresses caused by apoptotic stimulation, since JNK activation is blocked by antioxidants, N-acetyl-cysteine and 2-mercaptoethanol. Bcl-2 suppresses JNK activation, suggesting that Bcl-2 is located in the upstream of the JNK pathway.

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Mi-Rong Chen 6-11-98

PI - Signature Date

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INTRODUCTION

Apoptosis is a unique biochemical and morphological pattern of cell death characterized by internucleosomal DNA cleavage, chromatin condensation, membrane blebbing, and cell fragmentation. Apoptosis is as important as cell proliferation in regulating the development and maintenance of homeostasis in multicellular organisms. Apoptosis is positively and negatively regulated by many physiological and pathological factors, and this regulation appears to be initiated by genetic and biochemical programs. Apoptosis occurs during the developmental process, and can be induced by ionizing radiation, UV radiation, DNA-damaging drugs, oxidants, viral infection, and deprivation of growth factors(1,2). Disorders in apoptosis may play a critical role in carcinogenesis or in the development of resistance to radiation or chemotherapy in cancer cells(2). Understanding the molecular mechanisms of the apoptotic process is important for the prevention and treatment of cancers.

JNK (also called stress-activated protein kinase, SAPK) family members belong to the mitogen-activated protein kinase (MAPK) superfamily which also includes extracellular signal-regulated kinases (ERKs) and the p38-MAPK family(3-6). MAPKs are serine/threonine kinases which target other kinases, transcription factors, and membrane receptor tails, causing diverse effects such as cell proliferation, transformation, differentiation, and apoptosis. Presently, the JNK family consists of three genes, JNK1, JNK2, and JNK3, which can be further subdivided into ten isoforms(7). Substrates for JNK family members include the transcription factors c-Jun(7-11), JunD(7), ATF-2(12-14), ATFa (15), Elk-1(14,16,17), Sap-1a(18), and p53(19,20); phosphorylation of c-Jun, ATF-2, Elk-1, and Sap-1a increases their transcriptional activity.

JNK kinase activity can be activated by proinflammatory cytokines (TNF-α and IL-1), G protein-coupled receptors, lymphocyte costimulatory receptors (CD28 and CD40), osmotic shock, heat shock, protein synthesis inhibitors, ceramides, DNA-damaging chemicals, UV radiation, and γ radiation (10,11,21-33). Activation of JNK involves MKK4 (also called SEK1 and JNKK; (34-36)) and MEKK1(37,38). MEKK1 phosphorylates and activates the dual-specificity kinase MKK4/SEK(39,40). MKK4/SEK then activates JNK via threonine and tyrosine phosphorylation of the T-P-Y motif on JNK(3,34-36). Recently, a novel kinase called MKK7 has been cloned and found to specifically activate JNK, but not p38-MAPK and ERKs(41,42).

JNK activation has been observed in apoptosis induced by growth factor withdrawal, UV-C, γ radiation, ceramide, heat shock, and DNA-damaging drugs(32,33,43-46). Activation of the JNK pathway can lead to cell death(33,43). Interference with the JNK pathway by the dominant-negative mutant of MEKK1, MKK4/SEK, or JNK1 suppresses apoptosis(33,43-45). JNK's substrate, c-Jun, is required for ceramide-induced apoptosis(47) and apoptosis of neuronal cells caused by NGF withdrawal(47,48). All these results indicate the importance of the JNK pathway in apoptosis. However, reports on the necessity of JNK in Fas-mediated apoptosis are controversial (33,49,50), and JNK activation induced by TNF-α is not required for apoptosis induction(51,52).

The JNK pathway participates in cellular responses to mitogens, stresses, and apoptotic agents. How does the JNK pathway integrate with cellular signaling to achieve these diverse functions? We have found that the induction of JNK in response to mitogenic and apoptotic signals have different activation patterns, transient versus persistent, respectively (32,33). Co-treatment of a tyrosine phosphatase inhibitor (sodium orthovanadate) and T-cell activation signals (phorbol 12-myristate 13-acetate [PMA] plus ionomycin) prolongs the JNK induction by T-cell activation agents and results in T-cell apoptosis(33). These results suggest that the duration of JNK activation may be the determining factor for the outcome of signaling. **The aims of this study are (i) to examine the role of the JNK pathway in proliferation apoptosis in breast cancer cells, (ii) to study the mechanism of JNK-mediated apoptosis, (iii) to identified genes involved in JNK-mediated apoptosis.**

BODY

A. Induction of JNK by Mitogenic and Apoptotic Stimuli in Breast Cancer Cells *(these experiments are related to specific aim #1 in the Statement of Work)*

Previously, we found that the induction of JNK in response to mitogenic and apoptotic signals in T cells have different activation patterns, transient versus persistent, respectively (32,33). Co-treatment of a tyrosine phosphatase inhibitor (sodium orthovanadate) and T-cell activation signals (PMA plus ionomycin) prolongs the JNK induction by T-cell activation agents and results in T-cell apoptosis(33). These results suggest that the duration of JNK activation may be the determining factor for the outcome of signaling. These experiments are to examine if JNK is differentially regulated by mitogenic and apoptotic stimuli in breast cancer cells.

Materials and Methods

Cell culture, reagents, and radiation treatments. MCF-7 breast cancer cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and streptomycin and penicillin. Epidermal growth factor was purchased from Gibco BRL. PMA and 5-Fluoro-Uracil (5-FU) were purchased from Sigma. UV irradiation was performed by using a UV Stratalinker 1800 (Stratagene). Gamma irradiation was performed using a Gammacell 1000 ^{137}Cs source (10 Gy/min).

Cell extract preparation and immunocomplex kinase assays. Whole cell lysates were prepared by suspending 2 x 10^6 cells in 200 µl lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothrietol [DTT], 2 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM Na_3VO_4). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysate was cleared by centrifugation at 15,000 g for 3 min, and the supernatant was stored at -80°C. Kinase assays were carried out as described(33) with modifications. Endogenous JNK1 was precipitated by incubation with anti-JNK1 antibody (Ab101) and protein A-agarose beads (Bio-Rad) in the lysis buffer at 4°C for 3 h. The precipitates were washed twice with the lysis buffer, twice with the LiCl buffer (500 mM LiCl, 100 mM Tris-Cl [pH 7.6], and 0.1% Triton X-100), and twice with kinase buffer (20 mM 4-morpholinepropane-sulfonic acid [MOPS; pH 7.6], 2 mM EGTA, 10 mM $MgCl_2$, 1 mM [DTT], 0.1% Triton X-100, and 1 mM Na_3VO_4), then mixed with 5 µg of GST-Jun(1-79), 15 µM of ATP, and 10 mCi of [γ-^{32}P]ATP in 30 µl of kinase buffer. The kinase reaction was performed at 30°C for 30 min, then terminated by adding SDS sample buffer. The reaction mixtures were boiled and analyzed by SDS-PAGE and autoradiography.

Results

Induction of JNK by mitogenic agents in MCF-7 cells

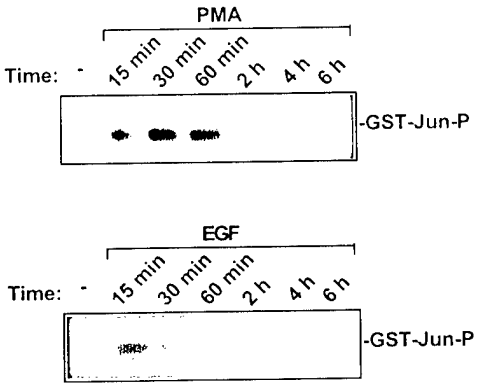

Fig.1 Induction of JNK by mitogenic agents in MCF-7 cells. MCF-7 cells were cultured in medium containing 1% serum for 12 h, then treated with either PMA (50 ng/ml) or EGF (10 ng/ml). Cells were collected at indicated time points and endogenous JNK was examined by immunocomplex kinase assays.

To examine the JNK induction by mitogenic agents in breast cancer cells, we treated MCF-7 cells with either PMA or epidermal growth factor (EGF). Both PMA and EGF induce an immediate and transient JNK activation in MCF-7 cells (Fig. 1). The kinase activity increased in 15 min and decreased to basal levels in 90 min.

Induction of JNK by UV-C and 5-FU, but not by γ radiation in MCF-7 cells

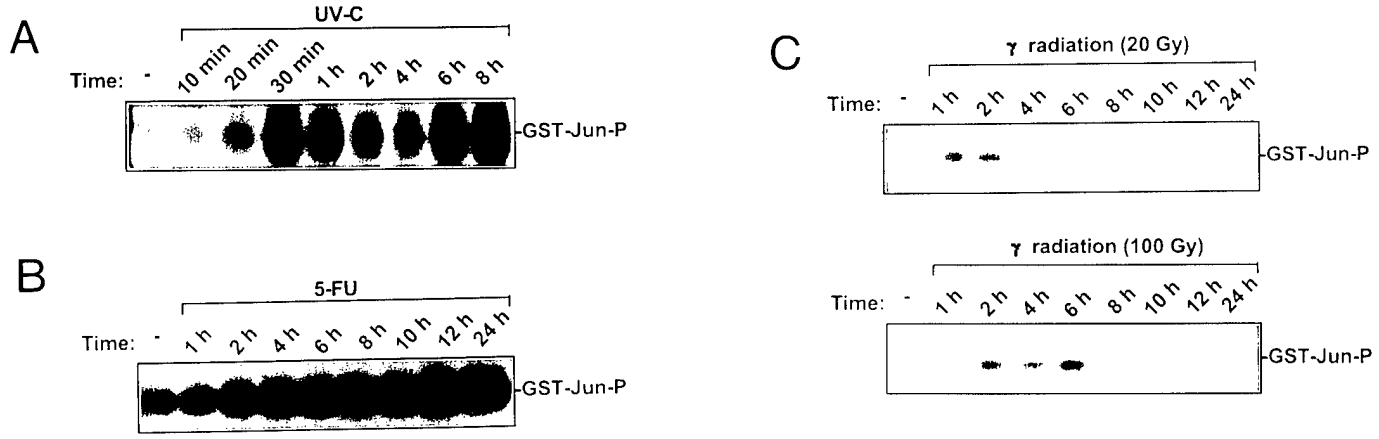

Fig.2 Induction of JNK by apoptotic agents in MCF-7 cells. MCF-7 cells were treated with (A) UV-C (200J/m^2), (B) 5-FU (250 μM), or (C) γ radiation(20 or 100 Gy). Cells were collected at indicated time points and endogenous JNK was examined by immunocomplex kinase assays.

MCF-7 were treated with various apoptotic agents, and examined for endogenous JNK activation. Among the agents tested, UV-C and 5-FU showed strong JNK inducing ability. Both agents induced persistent JNK activation (Fig. 2, A and B), which followed by apoptosis as determined by morphological change and nuclear straining of the cells (data not shown). To our surprise, γ radiation induced only weak JNK activation in MCF-7 cells (Fig. 2C). We also did not detect apparent apoptosis induction in MCF-7 cells by propidium iodide staining of the nuclei (less than 5% condensed nuclei) 48 h after irradition.

Discussion

These data reveal that mitogenic agents induced transient JNK activation in MCF-7 cells, while apoptotic agents induced persistent JNK activation. These results are consistent with our previous observation in T cells (32,33), and further support our hypothesis that duration of JNK activation may determine cell fate. We were unable to induced significant JNK activation and apoptosis induction in MCF-7 with γ radiation. Currently, we are testing the JNK activation by other apoptotic agents, such as isothiocyanates and adriamycin. Next, we will modulate the duration of JNK activation using inducible system for JNK and dominant-negative JNK in combination with either mitogenic or apoptotic signals to test our hypothesis.

B. Tumor Suppressor p53, Death Receptor Fas, and JNK-mediated Apoptosis *(these experiments are related to specific aim#2 in the Statement of Work)*

Tumor suppressor p53 is important for apoptosis induced by γ radiation and by adenovirus E1A protein(53,54). Since JNK phosphorylates both murine and human p53 proteins *in vitro*(19,20), it has been

suggested that p53 is a downstream effector of the JNK pathway. Fas and Fas ligand (FasL), which are important in apoptosis induction, can be induced by γ radiation and DNA-damaging drugs(55,56). The induction of Fas/FasL has been suggested to be involved in apoptosis induced by those agents. These experiments are to examine the relationship between p53, Fas expression, and JNK activation.

Materials and Methods

Cells, antibodies, and plasmid. Prostate carcinoma cell lines, LNCaP and PC-3 (from ATCC), and myeloid leukemia cell lines, HL-60, BV173, and KBM7 (provided by Dr. W. Zhang, M. D. Anderson Cancer Center, Houston) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and streptomycin/penicillin. Rabbit anti-JNK1 antibody (Ab101) was described previously(32). Anti-Fas and anti-FasL antibodies were purchased from Pharmingen. GST-Jun(1-79) and HA-JNK plasmids were described previously(32,57). pcDNA3-Flag-JNK1(APF) was provided by R. J. Davis (U. of Massachusetts, Worcester, MA).
DNA fragmentation and nuclear staining assays. For the DNA fragmentation analysis, 10^6 cells were lysed in 50 μl of NTE buffer (100 mM NaCl, 40 mM Tris-Cl [pH7.4], 20 mM EDTA) containing 0.5% SDS. The lysate was heated at 65°C for 10 min for inactivation of nucleases, and then digested with 0.5 mg/ml of proteinase K at 50°C for 2 h. The lysate was then incubated with 0.2 mg/ml RNase A at 50°C for 2 h. The DNA fragmentation was analyzed on a 1.8% agarose gel in the presence of 0.5 μg/ml ethidium bromide. For nuclear morphology staining, the harvested cells were fixed with 1% paraformaldehyde (in 1X PBS) for 10 min, washed once with 1X PBS, then incubated in Hoechst 33258 (2.5 ng/ml in PBS) for staining. The nuclear morphology was examined by a fluorescence microscope.
Transient Transfection Apoptosis Assays. For transient transfection-apoptosis assays, LNCaP or PC-3 cells were transfected with plasmids encoding β-galactosidase (3 μg) in combination with indicated kinase plasmids as described in figure legends. Cells were fixed in 1% paraformaldehyde for 10 min, washed twice with PBS, and stained with X-gal staining. Transfected cells (blue color) with round up, shrinking, or membrane blebbing morphology were identified as apoptotic cells. Apoptosis induction was represented as *% of apoptotic cells in 200 blue cells.*
Flow cytometry analyses. One million cells with or without treatments were harvested and stained with FITC-conjugated anti-Fas antibody (20 μl) in 100 μl of 1X phosphate buffered saline (PBS) containing 2% of fetal calf serum (FCS) on ice for 30 min. The stained cells were then washed twice with PBS containing 2% FCS, and fixed with 1% paraformaldehyde in PBS. Surface staining for FasL was carried out similarly by using biotin-conjugated anti-FasL antibody followed by FITC-conjugated avidin. Cell staining was analyzed by flow cytometry assays (Profile and XL, Coulter Co.).

RESULTS

Tumor suppressor p53 is not required for radiation-induced JNK activation.

To study the necessity of p53 in JNK activation, we examined the JNK activation in two prostate carcinoma cell lines with different p53 status, $p53^{+/+}$ LNCaP cells and $p53^{-/-}$ PC-3 cells(58,59). We used γ radiation and UV-C to activate the JNK pathway. Both γ radiation (20 Gy) and UV-C (100 J/m^2) induced JNK activation in PC-3 cells as well as in LNCaP cells (Fig. 3 A and B). The radiation-induced apoptosis was detected by examination of morphological changes of the cells 24-48 h after treatments (data not shown). This result shows that the JNK pathway is responsive to the radiation treatments in both $p53^{+/+}$ and $p53^{-/-}$ cells.

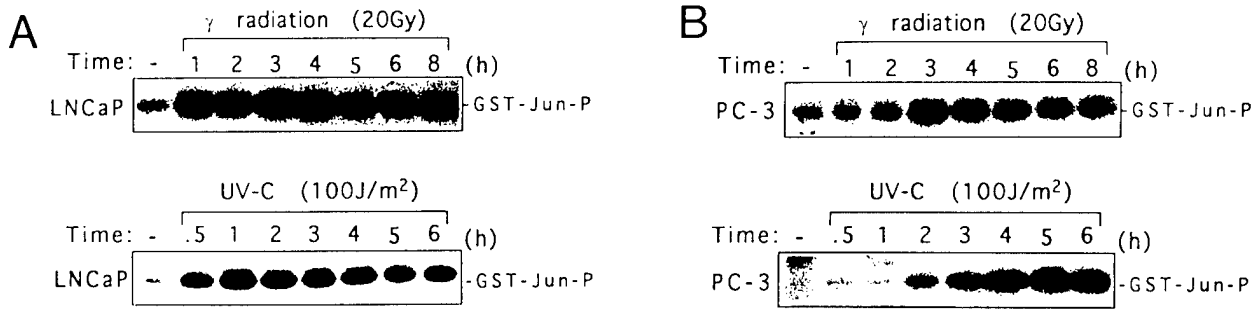

Fig. 3. Both LNCaP and PC-3 cells have sustained JNK activation after radiation treatments. LNCaP (A) and PC-3 (B) cells were treated with γ radiation(20 Gy) or UV-C (100 J/m^2). The cells were harvested at indicated time points and the endogenous JNK activity was determined by immunocomplex kinase assays as described in Materials and Methods.

Activation of the JNK Pathway Induces Apoptosis in both LNCaP and PC-3 cells.

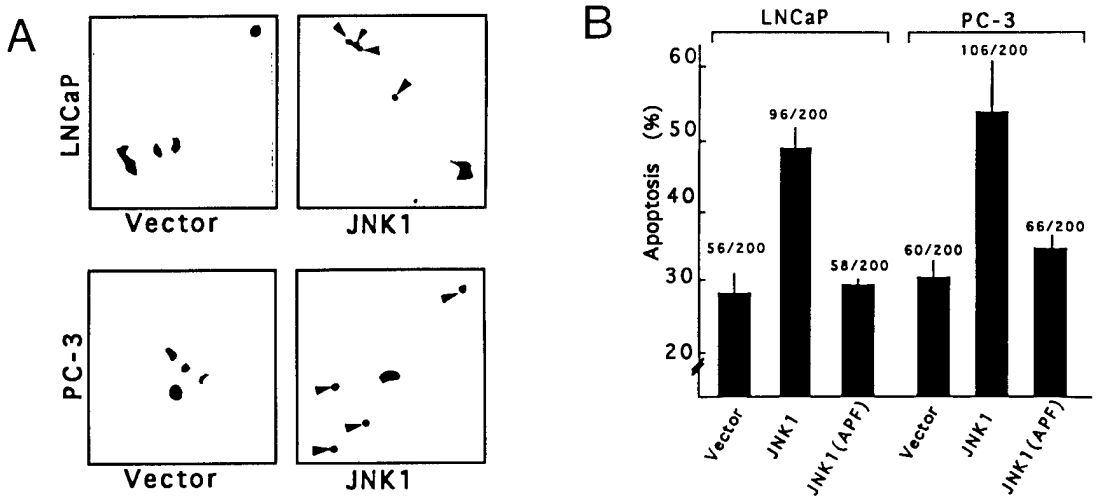

Fig. 4. Overexpression of JNK1 induces apoptosis in both LNCaP and PC-3 cells. LNCaP and PC-3 cells were transfected with plasmids encoding β-galactosidase (3 μg) in combination with the indicated plasmids (HA-JNK1, 2 μg; JNK1 [APF], 2 μg). Total amounts of transfected DNA were made even with empty vectors. (**A**) Cells were stained with X-gal either 24h (PC-3) or 48 h (LNCaP) after transfection. Transfected cells (blue color) with rounding up, shrinking, or membrane blebbing morphology were identified as apoptotic cells (indicated by arrow heads). (**B**) Apoptosis induction was represented as *% of apoptotic cells per 200 blue cells*. Data represent the means and standard deviations of four experiments.

It has been shown that activation of the JNK pathway is sufficient to induce apoptosis(33,43,60). When we transfected a JNK1 plasmid or the empty vector into LNCaP and PC-3 cells, we observed an increase in apoptosis in the JNK1-transfected cells in comparison with the control (Figure 4, A and B). In contrast, the kinase-dead JNK1 (JNK1[APF]) failed to induce apoptosis (Figure 4B). These data demonstrate that both LNCaP and PC-3 cells can undergo apoptosis following activation of the JNK pathway in the absence of other signals.

Induction of Fas expression is associated with the status of p53 but not with the JNK activation.

To examine the relation between JNK activation and Fas expression, surface expression of Fas was examined in cells treated with radiation. We examined the Fas expresssion in p53$^{+/+}$ MCF-7 cells after radiation

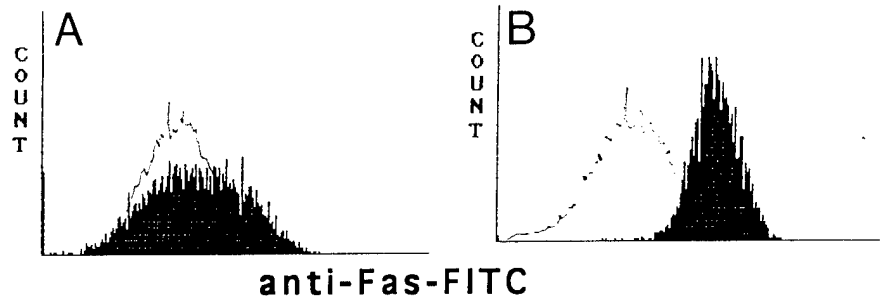

Fig.5. MCF-7 cells were treated with either (A) UV-C (200 J/m^2) or (B) γ radiation (20 Gy). Surface expression of Fas was determined by flow cytometry assays (light area, untreated cells; shaded area, treated cells)

treatments. Although UV-C induced strong JNK activation in MCF-7 cells (Fig. 2A), it only marginally increase Fas expression on cell surface (Fig. 5A). In contrast, γ radiation significantly ehance Fas expression (Fig. 5B), although it did not significantly induce JNK activity in MCF-7 cells (Fig. 2C).

γ radiation induced JNK activation and apoptosis in both LNCaP and PC-3 cells; however, γ radiation caused Fas expression only in LNCaP cells (a $p53^{+/+}$ cell line) but not in PC-3 cells (a $p53^{-/-}$ cell line) (Fig. 6A), suggesting that fas expression may associate with a wild-type p53 status. To further study this issue, We examined the effect of γ radiation on three additional cell lines with different p53 status ((61); Zhang W., personal communication). γ radiation induced JNK activation (Fig. 6B) and apoptosis (data not shown) in three myeloid cells lines, HL-60, KBM-7, and BV173, although KBM-7 cells were more resistant to γ radiation in JNK activation and apoptosis induction than the other two. Nevertheless, γ radiation only increase Fas expression levels in BV-173 which have wild type p53 genes, but not in $p53^{-/-}$ HL-60 cells or in KBM-7 cells which have mutated p53 (Fig. 6C). The induction of Fas expression was also irrelevant to the basal expression levels of Fas in each cell lines.

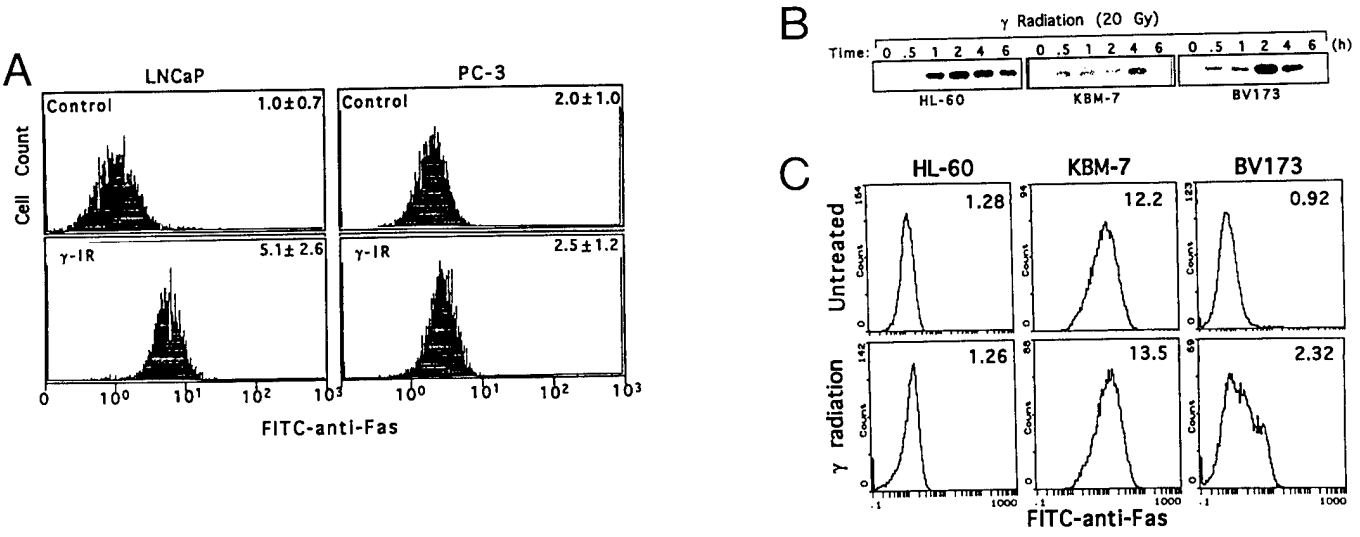

Fig. 6. Induction of Fas expression was associated with the status of p53 but not with the JNK activation. A, LNCaP and PC-3 cells were treated with or without γ radiation(20 Gy). Cells were harvested 20 h after treatments, and stained with FITC-conjugated anti-Fas antibody. The expression levels of Fas were examined by flow cytometry assays. B, HL-60, KBM-7, and BV173 cells were irradiated with γ radiation (20 Gy). The cells were collected at different time points, and the endogenous JNK activities were analyzed by immunocomplex assays. C, HL-60, KBM-7, and BV173 cells were irradiated with or without γ radiation (20 Gy). HL-60, KBM-7, and BV-173 were harvested 8 h after radiation. Surface expression of Fas on irradiated cells were examined as described in Materials and Methods.

Discussion

Since JNK can be activated in $p53^{-/-}$ PC-3 cells by radiation we conclude that the p53 is not require for JNK activation, at least it is not required for radiation-induced JNK activation. Our results reveal that the apoptosis signaling downstream of the JNK pathway is functional in the $p53^{-/-}$ PC-3 cells, because overexpression of JNK induces apoptosis in transfected PC-3 cells. This indicates that p53 is not required for JNK-induced apoptosis. However, the data did not exclude the possibility that p53 may participate in JNK signaling. Our data also indicate that the induction of Fas expression is associated with the presence of functional p53 proteins, and JNK activation is not necessarily associated with surface expression of Fas receptors.

C. Oxidative Stresses, Bcl-2, and JNK-mediated Apoptosis *(these experiments are related to specific aim#2 in the Statement of Work; also see the appended reprint)*

The JNK pathway has been shown to be activated by many apoptotic stimuli; however, the mechanisms by which it was activated are unclear. Intracellular oxidative stress is one major event in apoptotic cells. Antioxidants are shown to have protective effects aganinst many apoptotic stimuli. Bcl-2 or Bcl-X_L are important anti-apoptotic regulators. It has been shown that Bcl-2 may protect cells by preventing oxidative damages(62,63). In the following experiments, we use pro-oxidative and anticarinogenic isothiocyanates (64-68)as a probe to examine the mechanism of JNK activation and the interaction between the JNK pathway and Bcl-2 family proteins.

Materials and Methods

Cells, Antibodies, Plasmids, and Reagents. Human Jurkat T cells (clone J.LEI) were cultured as described(32). HeLa cells and human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% fetal calf serum and streptomycin/penicillin. Rabbit anti-JNK1 antiserum (Ab101) was described previously(32). Anti-Bcl-2 (#100; mouse mAb), anti-Bcl-x_L (#S-18; rabbit Ab), and the goat anti-mouse (horse radish peroxidase [HRP]-conjugated) antibodies were purchased from Santa Cruz. The goat anti-rabbit (HRP-conjugated) antibody was obtained from Sigma. GST-Jun(1-79) plasmid, pCIneo-JNK1, pCMV-ΔMEKK1, pUna3-MEKK1-KR, pcDNA3-Flag-JNK1(APF), pCMV-Flag-p38(AGF), and Raf-BXB-301 plasmid were described previously(33,57,69). Bcl-2 and Bcl-x_L expressing vectors were obtained from Dr. D. Spencer (Baylor College of Medicine, Houston, TX). The caspase/interleukin-1 b converting enzyme (ICE) inhibitor, Z-VAD-FK, and anti-Fas antibody (CH-11) were purchased from Kamiya Biomedical. Phenyl isothiocyanate (PITC), phenylmethyl isothiocyanate (PMITC), and phenylethyl isothiocyanate (PEITC) were purchased from Fluka. Phenylpropyl isothiocyanate (PPITC), PMA, ionomycin, and anisomycin were purchased from Sigma.

Transient Transfection-Protection/Death Assays. The protection assay was performed as described (70) with modifications. Briefly, 293 cells were plated 24 h before transfection at a density of 1.5×10^5 per 35-mm well. Cells were co-transfected with pCMV-βGal plasmid encoding b-galactosidase and plasmids for control vector, Bcl-2 (or Bcl-x_L), or mutant kinases by a calcium phosphate precipitation protocol (Specialty Media), with duplicates in each transfection. After removing the transfection mixture, the cells were incubated in complete medium for 12 h for recovery, then treated with or without drugs. Cells were harvested 24 h post-treatment, washed and fixed in 1% paraformaldehyde in phosphate buffer saline (PBS). The fixed cells were washed once with PBS and resuspended in staining solution (PBS [pH 7.4], 1 mM $MgCl_2$, 10 mM $K_4[Fe(CN)_4]$, 10 mM $K_3[Fe(CN)_4]$, 0.1% Triton X-100, and 1 mM X-gal) for 2-6 h, then washed twice with PBS. The ratio of b-galactosidase-expressing cells (blue color) was examined with a hemacytometer. Cell survival was determined as: *(% of blue cells in treated group/% of blue cells in untreated group) x 100%.*

To perform transient transfection-cell death assays, 293 cells were transfected with empty vectors or JNK1

plus ΔMEKK1 expressing plasmids (in the absence or presence of Bcl-2 [or Bcl-x_L]). Cells were collected 48 h after transfection, fixed, and stained as described above. Cell survival was determined as: *% of blue cells in the experimental group/ % of blue cells in the control) x 100%.*

Western Blot Analysis. Cells were lysed in lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10 % glycerol, 1 mM DTT, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 1 mM Na_3VO_4). The lysate was resolved by SDS-PAGE (12%), and then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with primary antibody (anti-CPP32, 1:200; anti-Bcl-2, 1:500 dilution; anti-Bcl-x_L, 1:1,000 dilution), washed, and blotted with secondary antibody conjugated with HRP (1:1,000 dilution). The membrane was then developed in ECL reagent (Amersham) and exposed to X-ray film.

Results

Anticarcinogenic isothiocyanates, PMITC and PEITC, induce sustained JNK activation and apoptosis

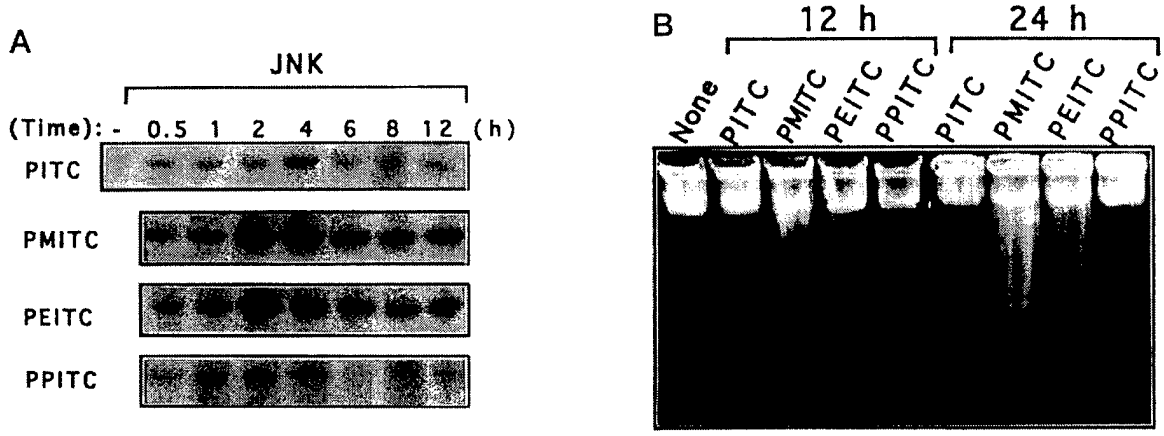

Fig. 7. Persistent JNK activation and apoptosis induced by PMITC and PEITC. A, Jurkat cells were treated with different isothiocyanates (5 μM), then cells were collected at indicated time points and endogenous JNK activity was determined by the immunocomplex assay. B, Jurkat cells were treated with different isothiocyanates (5 μM), and collected at 12-h and 24-h time points. Cellular DNA was extracted and analyzed on a 1.8% agarose gel for DNA fragmentation.

To examined the JNK inducing ability of isothiocyanates, Jurkat T cells were subjected to different isothiocyanates. Both PMITC and PEITC induced persistent JNK activation. JNK activity increased at the 1-h time point, peaked around 2-4 h post-treatment, and gradually decreased, but remained higher than basal levels even 12 h after treatment (Fig. 7A). In contrast, 5 μM of PITC or PPITC failed to induce any JNK activation. In our previous study, persistent JNK activation was associated with apoptosis induced by γ radiation and UV-C (33). Hence, a DNA fragmentation assay was used to examine if the persistent JNK activation induced by PMITC and PEITC were associated with apoptotic cell death. Cellular DNA was extracted from Jurkat cells treated with different isothiocyanates (5 μM), and analyzed for DNA fragmentation. We found that only PMITC and PEITC, which induced persistent JNK activation, caused chromosomal DNA laddering at 12-24 h after treatment (Fig. 2B). This result shows the correlation between persistent activation of JNK and apoptosis induction by isothiocyanate treatments, and the precedence of JNK activation to DNA fragmentation.

PEITC induces JNK activation and apoptosis in various cell types

We next examined if the isothiocyanate induces persistent JNK activation and apoptosis in different cell

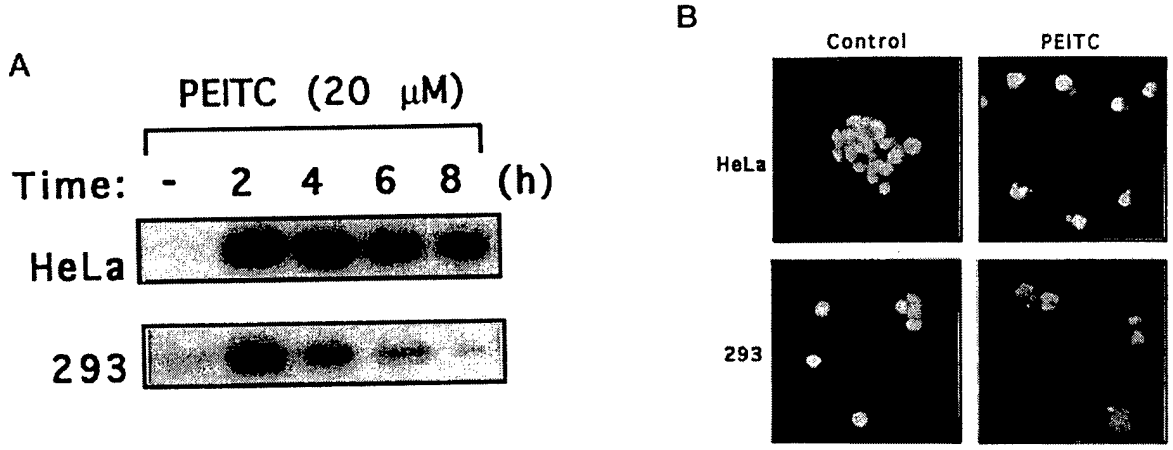

Fig. 8. JNK induction and apoptosis induced by PEITC in HeLa and 293 cells. A, HeLa and 293 cells were treated with PEITC (20 μM), and the treated cells were harvested at different time points as indicated. Endogenous JNK activity was examined by immunocomplex assays. B, HeLa and 293 cells were treated with or without PEITC (20 μM) 24 h, then the cells were harvested, washed once with PBS, and fixed in 1% paraformaldehyde in PBS. The fixed cells were then incubated with Hoechst 33258 (2.5 μg/ml in PBS). Nuclear staining was examined by a fluorescence microscope.

types. We found that different concentrations of PEITC were needed to induce JNK activation in distinct cell types; the effective concentration varied from 5-50 μM (data not shown). Twenty μM of PEITC induced persistent JNK activation in HeLa and 293 cells (Fig. 8A). At the concentration that induced sustained JNK induction, PEITC also caused apoptosis (Fig. 8B). Examining by Hoechst 33258 staining, the cell nuclei were condensed and fragmented after the PEITC treatment, in comparison with the homogeneous nuclear staining of the untreated cells (Fig. 8B). These data demonstrate that the induction of JNK activation and apoptosis by PEITC can occur in various cell types.

Interfering with the JNK pathway suppresses PEITC-induced apoptosis

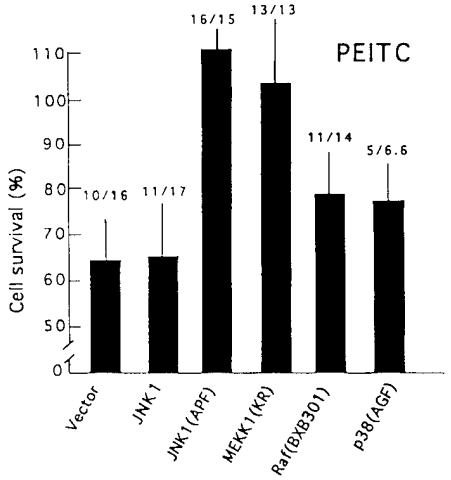

Fig. 9. Suppression of PEITC-induced apoptosis by interfering with the JNK pathway. 293 cells were transfected in duplicates with pCMV-βGal (1 μg) and mutant kinase-expressing vectors (3 μg for each) as indicated. Empty vectors were used to make the total transfected DNA to 7 μg. After transfection, the cells were incubated in culture medium for 12 h, and then treated with or without PEITC (20 μM). Cells were harvested 24 h after treatment and the b-gal positive (blue) cells were examined by enzymatic staining using X-gal as a substrate. Cell survival was determined as *(% of blue cells in treated group/% of blue cells in untreated group)* x 100%. Data presented were the mean and standard deviation of six experiments.

293 cells were transfected with pCMV-βGal, with or without plasmids encoding a dominant-negative kinase mutant, and each transfection was duplicated for treatments with or without PEITC (20 μM). The cells were harvested 24 h after treatment, and stained by X-gal to examine the b-galactosidase-expressing cells (blue in color). The survival rate of transfected cells after the drug treatment was determined as the percentage of blue cells in the treated group divided by the percentage of blue cells in the untreated group. The dominant-negative mutants of MEKK1 and JNK1 (MEKK1[KR] and JNK1[APF], respectively), blocked PEITC-induced cell death in transfected 293 cells (Fig. 9). In contrast, transfection of wild-type JNK1, dominant-negative Raf1 mutant (Raf-BXB301), or

dominant-negative p38-MAPK (p38[AGF]) did not significantly affect apoptosis induced by PEITC. This result indicates that interfering with the JNK pathway prevents the PEITC-induced apoptosis, thereby suggesting, that the JNK pathway is required for isothiocyanate-induced apoptosis.

Isothiocyanate-induced JNK activation is inhibited by antioxidants

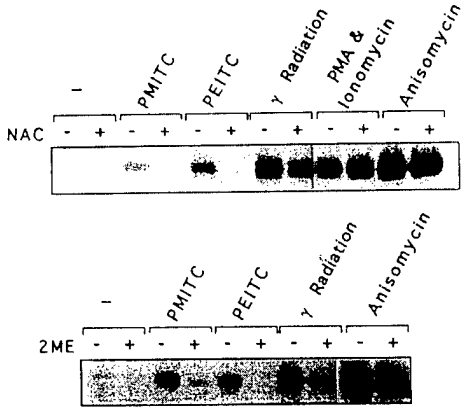

Fig. 10. Inhibition of JNK activation by antioxidants. Jurkat cells were pretreated with or without *N*-acetyl-$_L$-cysteine (NAC, 20 mM) or 2-mercaptoethanol (2ME, 10 mM), then treated with different stimuli: PMITC (5 μM, 2 h), PEITC (5 μM, 2 h), γ **radiation** (100 Gy, 2 h), PMA (50 ng/ml) plus ionomycin (1 μM), or anisomycin (2 μM, 30 min). JNK activity was assayed by immunocomplex kinase assays.

Isothiocyanates react with reduced glutathione (GSH) and form dithiocarbamates [R-NH-C(=S)-SG] in the presence or absence of glutathione S-transferase (GST)(71). Since apoptosis is activated by many oxidative agents(72), the isothiocynates may induce oxidative stress by reacting with and depleting the intracellular GSH pool, which may then induce JNK activation and apoptosis. By preincubation of the Jurkat cells with antioxidants, 2-mercaptoethanol (10 mM; 1 h) or N-acetyl-cysteine (20 mM; 2 h), JNK activation by PMITC and PEITC were inhibited (Fig. 10). The antioxidant treatments also blocked γ radiation-induced JNK activation, but had no effect on JNK induction by PMA plus ionomycin or by anisomycin (Fig. 10). The inhibition of JNK activation is not due to the loss of viability of Jurkat cells after antioxidant treatment, because over 95% of the cells retained the ability to exclude trypan blue 6 h after antioxidant treatment (data not shown). These data indicate that the JNK activation in cells exposed to PMITC, PEITC, or γ radiation may be due to the induction of oxidative stresses.

Bcl-2 suppresses PEITC-induced JNK activation and apoptosis

Bcl-2 family members are known to be important apoptosis regulators(73). We used a transient transfection-cell death protection assay to test if Bcl-2 (or Bcl-x_L) can block apoptosis induced by isothiocyanates. The levels of the Bcl-2 (Bcl-x_L) protein were examined by Western blot analysis (Fig. 11A), revealing the production of transfected genes. The empty vector-control group lost 40% of cells after the drug treatment. In comparison, transfection of Bcl-2 or Bcl-x_L prevented most of the transfected cells from PEITC-induced apoptosis (Fig. 11A), indicating that Bcl-2 and Bcl-x_L were capable of protecting cells from PEITC-induced apoptosis. We also examined the PEITC-induced JNK activation in 293 cells transfected with empty vector or Bcl-2 encoding plasmids. Although only 40-50% of the cells were transfected, the endogenous JNK activity induced by PEITC was evidently decreased in Bcl-2 transfected cells (Fig. 11B). This result implicates Bcl-2 as an upstream suppressor to prevent JNK activation by apoptotic stimuli.

To further examine the molecular ordering between Bcl-2 (or Bcl-x_L) and the JNK pathway, we co-transfected Bcl-2 or Bcl-x_L with JNK1 plus constitutively active MEKK1(ΔMEKK1) into 293 cells. The transfection of JNK1 plus ΔMEKK1 led to persistent JNK activation and caused apoptosis independent of the upstream signals

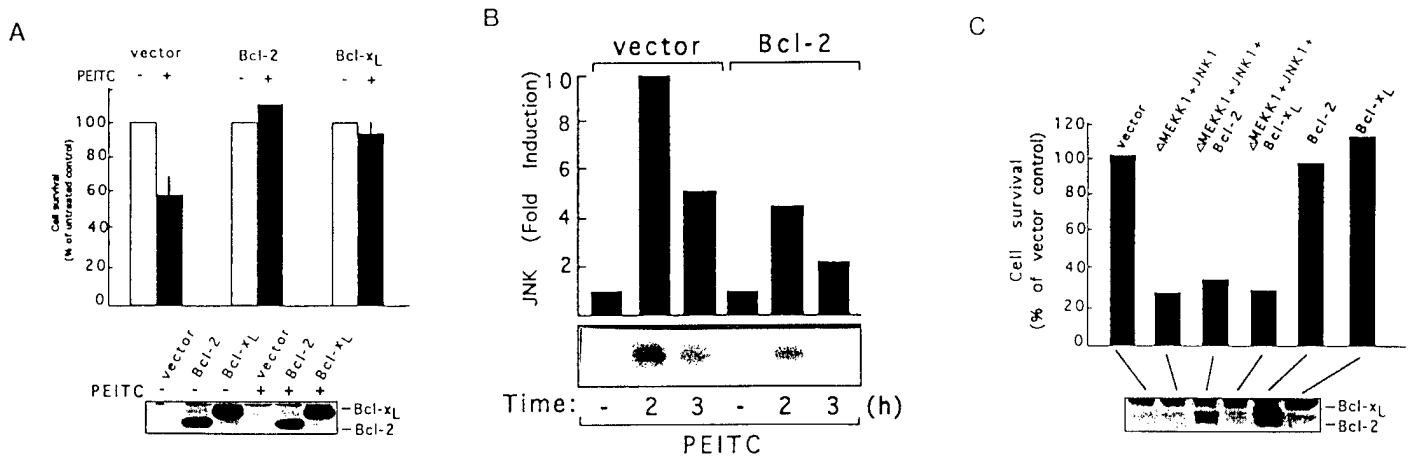

Fig. 11. Suppression of PEITC-induced JNK activation and apoptosis by Bcl-2. A, 293 cells were transfected with pCMV-βGal (1 μg) and control vector (3 μg) or expressing plasmid for Bcl-2 or Bcl-x_L (3 μg). Cells were cultured in complete medium for 12 h after transfection, then treated with or without PEITC (20 μM) for 24 h. Part of the harvested cells were assayed for cell survival as described in Materials and Methods. Data presented were the mean and standard deviation of three experiments. The remaining cells were lysed and the expressions of Bcl-2 and Bcl-x_L were determined by Western blot analysis. B, 293 cells were transfected with control vector or Bcl-2 encoding plasmid (3 μg), and transfected cells were treated with 20 μM of PEITC 6 h after removing the transfection mixture for indicated times. Endogenous JNK activity was examined by immunocomplex kinase assays. C, 293 cells were transfected with pCMV-βGal (1 μg) plus different combination of plasmids as indicated (JNK1, 1.5 μg; ΔMEKK1, 1.5 μg; Bcl-2 or Bcl-x_L, 3 μg). Empty vector was added to normalize the total DNA amount. Cells were collected 48 h after transfection, fixed, stained, and examined as described in Materials and Methods. Data of cell survival presented were the mean of three experiments. Western blots for Bcl-2 and Bcl-x_L were performed as described in Materials and Methods.

(Fig. 11C). Neither Bcl-2 nor Bcl-x_L significantly affected the cell death caused by JNK1 plus ΔMEKK1, although the levels of BCl-2 and Bcl-x_L were increased in the transfected cells (Fig. 11C). In comparison to the Bcl-2 (or Bcl-x_L) transfected cells, the lower levels of Bcl-2 and Bcl-x_L detected in the cells co-transfected with JNK1 plus ΔMEKK may be due to the loss of transfected cells caused by apoptosis (Fig. 11C). This result indicates that Bcl-2 and Bcl-x_L failed to prevent cell death induced by JNK activation; therefore, they may not be downstream of JNK in the apoptosis signaling pathway. Our results suggest that Bcl-2 and Bcl-x_L is upstream, but not downstream, of JNK in apoptosis signaling induced by isothiocyanates.

Discussion

In this study, we demonstrate the involvement of JNK in anticarcinognic isothiocyanate-induced apoptosis by proving that interfering with the JNK pathway suppressed isothiocyanate-induced cell death. Our result indicates that oxidative stress may initiate JNK activation. The anti-apoptotic regulator Bcl-2 may inhibit apoptosis by suppressing the formation or the damaging effects of free oxygen species (ROS)(62,63), which are generated by many apoptotic agents(72). In this report, we showed that Bcl-2 suppressed PEITC-induced JNK activation. Bcl-2 (or Bcl-x_L) blocked apoptosis caused by PEITC, but failed to suppress apoptosis caused by over-expression of activated JNK1. In addition, a recent report showed that Bcl-2 blocks JNK activation induced by serum depletion or NGF withdrawal in PC-12 cells(74). Taken together, Bcl-2 may be an upstream suppressor of the JNK pathway acting to relieve the oxidative stress and prevent JNK induction and the initiation of apoptosis signals. The failure

of Bcl-2 (or Bcl-x_L) to block cell death induced by ΔMEKK plus JNK suggests that Bcl-2 may not be the downstream of JNK. However, these data do not exclude the possibility that JNK may directly or indirectly down-regulate Bcl-2's function, therefore, causing cell death.

CONCLUSIONS

Our data shown that JNK is differentially regulated by mitogenic and apoptotic stimuli in breast cancer cell line MCF-7. Mitogens PMA and EGF induce immediate and transient JNK activation, in contrast, UV-C and 5-FU induce persistent JNK activation. These results suggest that duration of JNK activation may also determine cell fate in breast cancer cells. We will further examine issue by specifically regulate JNK activation by establishing the inducible system for JNK or dominant-negative JNK.

Tumor suppressor p53 is not required for radiation induced JNK activation. p53 is also not required for apoptosis induced by JNK activation. However, our data does not exclude the possibility that p53 may mediate JNK-induced apoptosis, which needs to be further studied. Both JNK activity and Fas expression can be induced by γ radiation however, Fas expression is closely associated with a wild-type p53 status but not with the JNK activation. Since JNK activation does not depend on p53 and JNK-mediated apoptosis does not require p53, these results suggest that Fas is not the downstream target for the JNK pathway.

We demonstrate that JNK is activated through oxidative stresses caused by apoptotic stimulation, since JNK activation is blocked by antioxidants, N-acetyl-cysteine and 2-mercaptoethanol. Bcl-2 suppresses JNK activation, suggesting that Bcl-2 is located in the upstream of the JNK pathway.

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APPENDIX

1. Chen, Y.-R., W. Wang, A.-N.T. Kong, and T.-H. Tan. 1998. Molecular mechanisms of c-Jun N-terminal kinase (JNK)-mediates apoptosis induced by anticarcinogenic isothiocyanates. *J. Biol. Chem.* 273: 1769-1775.

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Molecular Mechanisms of c-Jun N-terminal Kinase-mediated Apoptosis Induced by Anticarcinogenic Isothiocyanates*

(Received for publication, March 3, 1997, and in revised form, July 27, 1997)

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Isothiocyanates have strong chemopreventive properties against many carcinogen-induced cancers in experimental animal models. Here, we report that phenylmethyl isocyacyanate (PMITC) and phenylethyl isothiocyanate (PEITC) induced sustained c-Jun N-terminal kinase (JNK) activation in a dose-dependent manner. The sustained JNK activation caused by isothiocyanates was associated with apoptosis induction in various cell types. An inhibitor of the caspase/interleukin-1β-converting enzyme blocked isothiocyanate-induced apoptosis without inhibiting the JNK activation, which suggests that JNK activation by isothiocyanates is an event that is independent or upstream of the activation of caspase/interleukin-1β-converting enzyme proteases. PEITC-induced apoptosis was suppressed by interfering with the JNK pathway with a dominant-negative mutant of JNK1 or MEKK1 (JNK1(APF) and MEKK1(KR), respectively), implying that the JNK pathway is required for apoptotic signaling. Isothiocyanate-induced JNK activation was blocked by the antioxidants 2-mercaptoethanol and *N*-acetyl-L-cysteine, suggesting that the death signaling was triggered by oxidative stress. Overexpression of Bcl-2 suppressed PEITC-induced JNK activation. In addition, Bcl-2 and Bcl-x_L suppressed PEITC-induced apoptosis, but failed to protect cells from death induced by overexpression of activated JNK1. These results suggest that Bcl-2 and Bcl-x_L are upstream of JNK. Taken together, our results indicate (i) that JNK mediates PMITC- and PEITC-induced apoptosis and (ii) that PMITC and PEITC may have chemotherapeutic functions besides their chemopreventive functions.

Apoptosis plays important roles in developmental processes, maintenance of homeostasis, and elimination of seriously damaged cells (1, 2). The aberrant regulation of apoptosis has been observed in many disorders such as neuronal diseases, AIDS, autoimmune diseases, and cancers (2). In addition, many therapeutic agents eliminate tumor cells by inducing apoptotic cell death (2). Therefore, understanding the mechanism of apoptosis has important implications in the prevention and treatment of many diseases.

Recent studies have identified c-Jun N-terminal kinases (JNKs[1]; also named stress-activated protein kinases) to be involved in cellular responses to various extracellular stimuli (3). The JNK subfamily, including JNK1, JNK2, and JNK3 in various isoforms, is a member of the mitogen-activated protein kinase family (4). JNK activation requires phosphorylation at a specific motif (TPY) by a dual-specificity kinase, MKK4 (mitogen-activated protein kinase kinase 4) (5–7). MKK4 itself is activated by the upstream kinase MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1) (8). JNK can be dephosphorylated and inactivated by dual-specificity phosphatases (9, 10). JNK phosphorylates transcription factors such as c-Jun, ATF-2, and Elk-1 and strongly augments their transcriptional activity (11–13). JNK activity is induced by mitogenic signals including growth factors (14), oncogenic Ras (15), CD40 ligation (16, 17), and T-cell activation signaling (18) as well as by environmental stresses such as protein synthesis inhibitors (19), osmotic shock (20), pro-inflammatory cytokines (21, 22), and shear stress (23). In addition, JNK activation is required for apoptotic signaling induced by growth factor withdrawal (24), UV-C (25, 26), γ-radiation (26), ceramide (27), heat shock, and DNA-damaging drugs (25). The general involvement of the JNK pathway in cellular responses to various stimuli underscores its importance. Furthermore, the mechanisms by which the JNK pathway is integrated into the diverse cell signaling network are intriguing. Our previous results suggest that the duration of JNK activation determines cell proliferation and apoptosis (26, 28).

Many isothiocyanates are effective chemopreventive agents against carcinogen-induced cancers in experimental animals. Isothiocyanates inhibit cancer formation in various tissues such as rat lung, esophagus, mammary gland, liver, small intestine, colon, and bladder cancers (29–33). Isothiocyanates inhibit carcinogenesis caused by different compounds, including nitrosamines and polycyclic aromatic hydrocarbons (33). Previous studies suggested that isothiocyanates may inhibit enzymes (*e.g.* cytochrome P-450 isoforms) that are required for the bioactivation of carcinogens (34, 35). In addition, isothiocyanates may increase the carcinogen excretion or detoxification by inducing the phase II detoxifying enzymes, including glutathione *S*-transferase (GST), quinone reductase, epoxide

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[1] The abbreviations used are: JNKs, c-Jun N-terminal kinases; GST, glutathione *S*-transferase; PMITC, phenylmethyl isothiocyanate; PEITC, phenylethyl isothiocyanate; PITC, phenyl isothiocyanate; PPITC, phenylpropyl isothiocyanate; ICE, interleukin-1β-converting enzyme; *Z*-VAD-FK, *Z*-Val-Ala-Asp-CH_2F; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; MOPS, 4-morpholinepropanesulfonic acid.

hydrolase, and UDP-glucuronosyltransferase (35–37). Here, we report that phenylmethyl isothiocyanate (PMITC; benzyl isothiocyanate) and phenylethyl isothiocyanate (PEITC) are capable of inducing persistent JNK activation in a dose-dependent manner. Our study indicates the involvement of JNK-mediated apoptosis in the anticarcinogenic functions of isothiocyanates.

MATERIALS AND METHODS

Cells, Antibodies, Plasmids, and Reagents—Human Jurkat T-cells (clone J.LEI) were cultured as described (28). HeLa cells and human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle' medium supplemented with 10% fetal calf serum and streptomycin/penicillin. Rabbit anti-JNK1 antiserum (Ab101) was described previously (28). Anti-Bcl-2 (mouse monoclonal antibody 100), anti-Bcl-x_L (rabbit antibody S-18), anti-CPP32 (L-18), and horseradish peroxidase-conjugated goat anti-mouse antibodies were purchased from Santa Cruz. The horseradish peroxidase-conjugated goat anti-rabbit antibody was obtained from Sigma. Plasmids GST-Jun-(1–79), pCIneo-JNK1, pCMV-ΔMEKK1, pUna3-MEKK1(KR), pcDNA3-Flag-JNK1(APF), pCMV-Flag-p38(AGF), and Raf-BXB301 were described previously (26, 38, 39). Bcl-2- and Bcl-x_L-expressing vectors were obtained from Dr. D. Spencer (Baylor College of Medicine, Houston, TX). The caspase/interleukin-1β-converting enzyme (ICE) inhibitor Z-VAD-FK and anti-Fas antibody (CH-11) were purchased from Kamiya Biomedical Co. Phenyl isothiocyanate (PITC), PMITC, and PEITC were purchased from Fluka. Phenylpropyl isothiocyanate (PPITC), phorbol 12-myristate 13-acetate, ionomycin, and anisomycin were purchased from Sigma.

DNA Fragmentation Assays—10^6 cells were lysed in 50 μl of 100 mM NaCl, 40 mM Tris-Cl (pH 7.4), and 20 mM EDTA containing 0.5% SDS. The lysate was heated at 65 °C for 10 min for inactivation of nucleases and digested with 0.5 mg/ml proteinase K at 50 °C for 2 h. The lysate was then incubated with 0.2 mg/ml RNase A at 50 °C for 2 h. The DNA fragmentation was analyzed on a 1.8% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

Transient Transfection Cell Death/Protection Assays—The protection assay was performed as described (40) with modifications. Briefly, 293 cells were plated 24 h before transfection at a density of 1.5×10^5/35-mm well. Cells were cotransfected with the pCMV-βgal plasmid encoding β-galactosidase and plasmids for control vector, Bcl-2 or Bcl-x_L, or mutant kinases by a calcium phosphate precipitation protocol (Specialty Media), with duplicates in each transfection. After removing the transfection mixture, the cells were incubated in complete medium for 12 h for recovery and then treated with or without drugs. Cells were harvested 24 h post-treatment, washed, and fixed in 1% paraformaldehyde in phosphate-buffer saline (PBS). The fixed cells were washed once with PBS, resuspended in staining solution (PBS (pH 7.4), 1 mM $MgCl_2$, 10 mM $K_4(Fe(CN)_4)$, 10 mM $K_3(Fe(CN)_4)$, 0.1% Triton X-100, and 1 mM X-gal) for 2–6 h, and then washed twice with PBS. The ratio of β-galactosidase-expressing cells (blue color) was examined with a hemocytometer. Cell survival was determined as follows: (% of blue cells in treated group/% of blue cells in untreated group) × 100%.

To perform transient transfection/cell death assays, 293 cells were transfected with empty vectors or JNK1- plus ΔMEKK1-expressing plasmids (in the absence or presence of Bcl-2 or Bcl-x_L). Cells were collected 48 h after transfection, fixed, and stained as described above. Cell survival was determined as follows: (% of blue cells in the experimental group/% of blue cells in the control) × 100%.

Cell Extract Preparation and Immunocomplex Kinase Assays—Whole cell lysate was prepared by suspending 5×10^6 cells in 150 μl of lysis buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysate was cleared by centrifugation at 15,000 × *g* for 3 min, and the supernatant was stored at −80 °C. Kinase assays were carried out as described (41) with modifications. Endogenous JNK was precipitated by incubation with anti-JNK antiserum (Ab101) and protein A-agarose beads (Bio-Rad) in incubation buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). The precipitates were washed twice with incubation buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl (pH 7.6), and 0.1% Triton X-100), and twice with kinase buffer (20 mM MOPS (pH 7.6), 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100, and 1 mM Na_3VO_4) and then mixed with 5 μg of GST-Jun-(1–79), 15 μM of ATP, and 10 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer. The kinase reaction was performed at 30 °C for 30 min and then terminated by adding SDS sample buffer. The reaction mixtures were boiled and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Western Blot Analysis—Cells were lysed in lysis buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). The lysate was resolved by SDS-polyacrylamide gel electrophoresis (12%) and then transferred to polyvinylidene difluoride membrane. The membrane was incubated with primary antibody (anti-CPP32, 1:200 dilution; anti-Bcl-2, 1:500; and anti-Bcl-x_L, 1:1000), washed, and blotted with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution). The membrane was then developed in ECL reagent (Amersham Corp.) and exposed to x-ray film.

RESULTS

Isothiocyanates Induce JNK Activation in a Dose-dependent Manner—Human leukemia Jurkat T-cells were treated with different concentrations of PITC, PMITC, PEITC, and PPITC. Cells were collected 2 h after treatment, and endogenous JNK activity was determined by immunocomplex kinase assays. Among the tested isothiocyanates, PMITC and PEITC induced strong JNK activation at a concentration of 5 μM, and PITC induced mild JNK induction at higher concentrations (50–100 μM). No apparent JNK activation was observed with PPITC treatment at all concentrations tested (Fig. 1*A*). In contrast to the JNK activation, decreases in JNK activity (in comparison with the basal levels in the untreated group) were observed in treatments with high concentrations (>50 μM) of PMITC, PEITC, and PPITC (Fig. 1*A*). The decreases in JNK activity in treatments with high doses of isothiocyanates may be due to the acute cytotoxicity of these drugs. Jurkat cells swelled and lost the ability to exclude trypan blue (Fig. 1*B*), and the protein recovery in the treated cell lysate was significantly decreased after treatments with high doses of PMITC, PEITC, and PPITC. These phenomena indicated that the integrity of the cell membrane was lost and that the cells died by membrane disintegration and cytolysis, which is reminiscent of necrosis. However, >80% of the cells retained the ability to exclude trypan blue 24 h post-treatment with different isothiocyanates at 5 μM (Fig. 1*B*).

PMITC and PEITC Induce Sustained JNK Activation and Apoptosis—To exclude the possibility that the differential regulation of JNK by isothiocyanates is due to the selected observation at the 2-h time point, we did a time course study of JNK activation with various isothiocyanates at either 5 μM (Fig. 2*A*) or 50 μM (Fig. 2*B*) in Jurkat T-cells. At a concentration of 5 μM, both PMITC and PEITC induced persistent JNK activation. JNK activity increased at the 1-h time point, peaked around 2–4 h post-treatment, and gradually decreased, but remained higher than basal levels even 12 h after treatment. In contrast, 5 μM PITC or PPITC failed to induce any JNK activation. At 50 μM, only PITC induced a slight JNK induction, whereas PMITC, PEITC, and PPITC decreased the basal levels of JNK activity. These results were consistent with the data in the dose-response experiment (Fig. 1*A*), showing that isothiocyanates induced JNK activation in a dose-dependent manner and also revealing that PMITC and PEITC were strong JNK activators, inducing sustained JNK activation at certain concentrations.

In our previous study, persistent JNK activation was associated with apoptosis induced by γ-radiation and UV-C (26). Since most of the early apoptotic cells maintain an intact cytoplasmic membrane, trypan blue staining is not an appropriate criterion for apoptosis. Hence, a DNA fragmentation assay was used to examine if the persistent JNK activation induced by PMITC and PEITC was associated with apoptotic cell death. Cellular DNA was extracted from Jurkat cells treated with different isothiocyanates (5 μM) and analyzed for DNA frag-

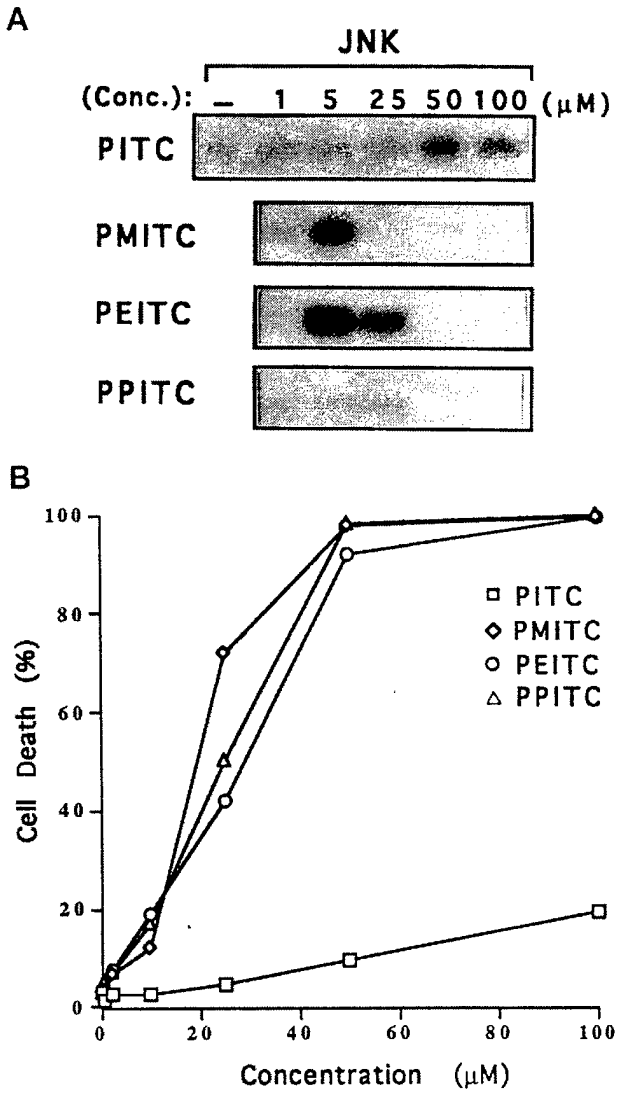

FIG. 1. **Dose response of Jurkat cells to isothiocyanates.** *A*, Jurkat cells were treated with different isothiocyanates in various concentrations as indicated. Cell lysate was collected 2 h after treatments, and endogenous JNK activity was determined by immunocomplex kinase assays using GST-Jun-(1–79) as a substrate. *B*, Jurkat cells were cultured in medium with or without different doses of isothiocyanates for 24 h, and then the percentage of dead cells was determined by the trypan blue exclusion assay.

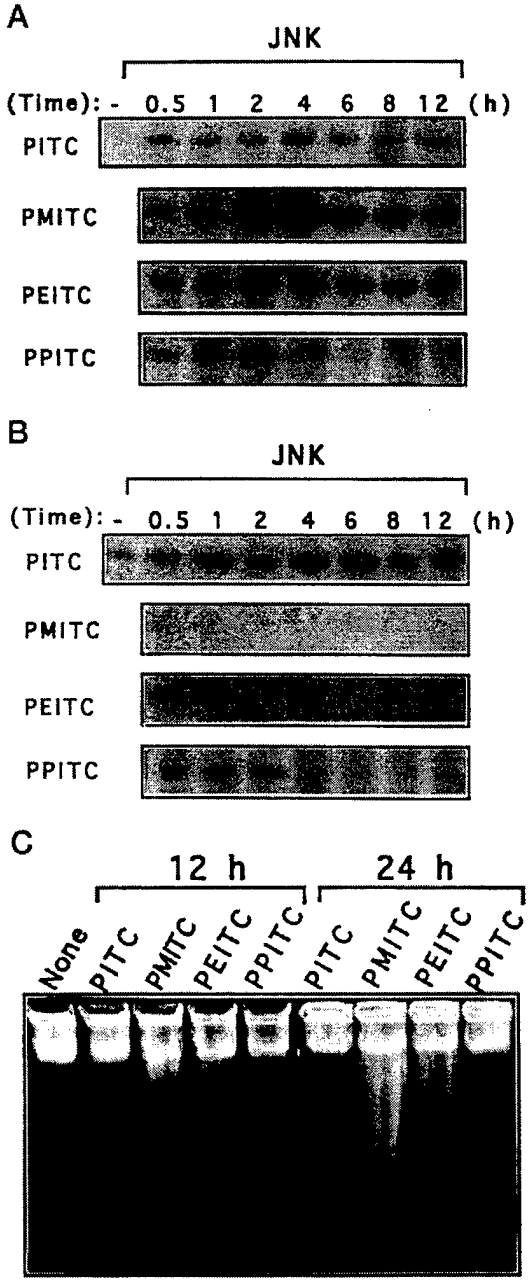

FIG. 2. **Persistent JNK activation and apoptosis induced by PMITC and PEITC.** *A* and *B*, Jurkat cells were treated with different isothiocyanates at either 5 μM (*A*) or 50 μM (*B*); cells were collected at the indicated time points; and endogenous JNK activity was determined by the immunocomplex assay. *C*, Jurkat cells were treated with different isothiocyanates (5 μM) and collected at 12- and 24-h time points. Cellular DNA was extracted and analyzed on a 1.8% agarose gel for DNA fragmentation.

mentation. We found that only PMITC and PEITC, which induced persistent JNK activation at 5 μM, caused chromosomal DNA laddering at 12–24 h after treatment (Fig. 2*C*). This result shows the correlation between persistent activation of JNK and apoptosis induction by isothiocyanate treatments and the precedence of JNK activation to DNA fragmentation.

The Caspase/ICE Protease Inhibitor Fails to Inhibit JNK Activation by Isothiocyanates—The caspase/ICE family of proteases are known to be important apoptosis mediators (42); hence, we determined if caspases/ICE-like proteases are involved in JNK-mediated apoptosis induced by isothiocyanates. PMITC- and PEITC-induced DNA fragmentation was completely inhibited by cotreatment with a caspase/ICE protease inhibitor, Z-VAD-FK (Fig. 3*A*). Z-VAD-FK blocked the cleavage of CPP32 (caspase 3) caused by anti-Fas treatment (Fig. 3*B*), indicating that it is an effective caspase inhibitor. In contrast to the inhibition of DNA fragmentation, Z-VAD-FK did not abolish JNK activation induced by PMITC (Fig. 3*C*) or PEITC (data not shown). The slight enhancement of JNK activation by Z-VAD-FK was not reproducible in repeated experiments, and therefore, was fluctuation of kinase assays. These data indicate the requirement of caspases/ICE-like proteases in isothiocyanate-induced apoptosis; however, JNK activation can occur in the absence of caspase activity. Also, in light of the fact that JNK activation occurred hours before the onset of DNA fragmentation during the isothiocyanate treatments (Fig. 2), JNK activation is unlikely to be a secondary effect of the cellular damage during apoptosis. These data suggest that JNK activation initiates apoptotic signaling.

PEITC Induces JNK Activation and Apoptosis in Various Cell Types—We next examined if the isothiocyanate induces

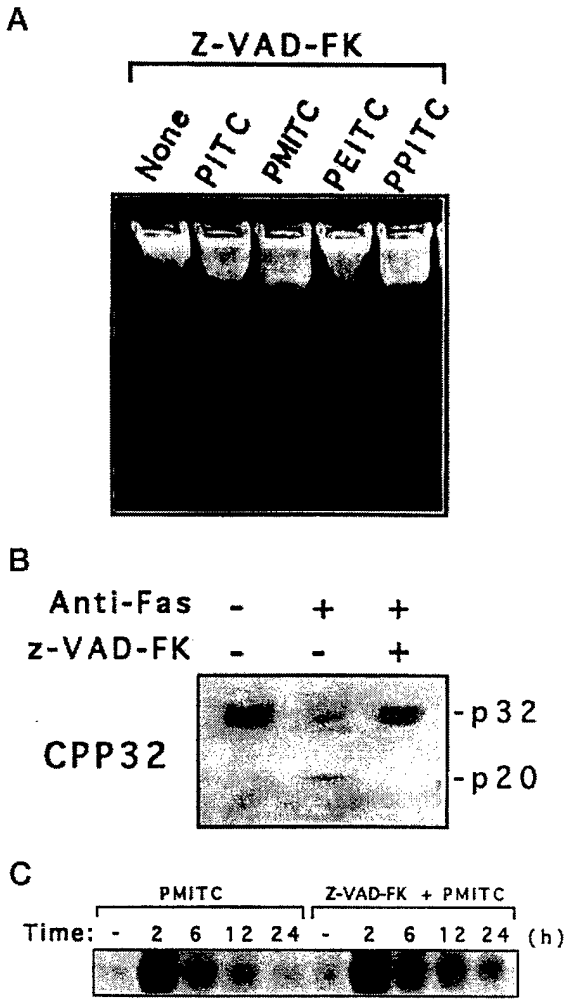

FIG. 3. **Failure of Z-VAD-FK to suppress isothiocyanate-induced JNK activation.** *A*, Jurkat cells were pretreated with the caspase/ICE inhibitor *Z*-VAD-FK (100 μM) for 3 h and treated with the different isothiocyanates at 5 μM for 24 h. The cells were then harvested and assayed for DNA fragmentation. *B*, Jurkat cells preincubated with *Z*-VAD-FK or with the solvent (dimethyl sulfoxide) were treated with anti-Fas antibody (CH-11; 100 ng/ml) for 4 h. The cleavage of CPP32 was examined by the Western blot analysis. *C*, Jurkat cells were pretreated with or without the caspase/ICE inhibitor *Z*-VAD-FK (100 μM) for 3 h. These cells were then treated with 5 μM PMITC. Samples of cells were collected at the time points indicated, and JNK activity was examined by immunocomplex assays.

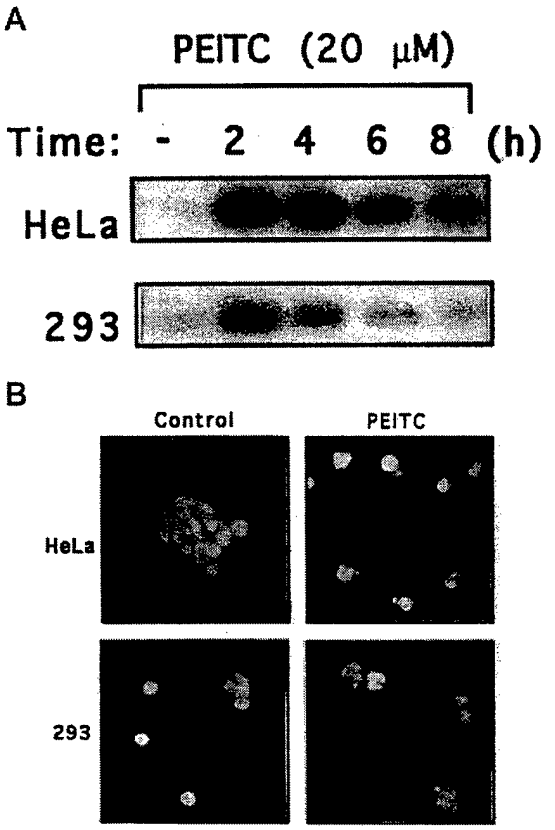

FIG. 4. **JNK induction and apoptosis induced by PEITC in HeLa and 293 cells.** *A*, HeLa and 293 cells were treated with PEITC (20 μM), and the treated cells were harvested at different time points as indicated. Endogenous JNK activity was examined by immunocomplex assays. *B*, HeLa and 293 cells were treated with or without PEITC (20 μM) for 24 h, and then the cells were harvested, washed once with PBS, and fixed in 1% paraformaldehyde in PBS. The fixed cells were incubated with Hoechst 33258 (2.5 μg/ml in PBS). Nuclear staining was examined with a fluorescence microscope.

persistent JNK activation and apoptosis in different cell types. We found that different concentrations of PEITC were needed to induce JNK activation in distinct cell types; the effective concentration varied from 5 to 50 μM (data not shown). 20 μM PEITC induced persistent JNK activation in HeLa and 293 cells (Fig. 4*A*). At the concentration that induced sustained JNK induction, PEITC also caused apoptosis (Fig. 4*B*). On examination after Hoechst 33258 staining, the cell nuclei were condensed and fragmented after the PEITC treatment, in comparison with the homogeneous nuclear staining of the untreated cells (Fig. 4*B*). These data demonstrate that the induction of JNK activation and apoptosis by PEITC can occur in various cell types.

Interfering with the JNK Pathway Suppresses PEITC-induced Apoptosis—Previously, we have shown that the JNK pathway is involved in and required for radiation-induced apoptosis (26). Other investigators also showed that the JNK cascade is required for apoptosis induced by growth factor withdrawal or ceramide treatment (24, 27). We then tested if interfering with the JNK pathway had a suppressive effect on isothiocyanate-induced apoptosis. 293 cells were transfected with pCMV-βgal with or without plasmids encoding a dominant-negative kinase mutant, and each transfection was duplicated for treatments with or without PEITC (20 μM). The cells were harvested 24 h after treatment and stained with X-gal to examine the β-galactosidase-expressing cells (blue in color). The survival rate of transfected cells after the drug treatment was determined as the percentage of blue cells in the treated group divided by the percentage of blue cells in the untreated group. The dominant-negative mutants of MEKK1 and JNK1 (MEKK1(KR) and JNK1(APF), respectively) blocked PEITC-induced cell death in transfected 293 cells (Fig. 5). In contrast, transfection of wild-type JNK1, a dominant-negative Raf1 mutant (Raf-BXB301), or dominant-negative p38 mitogen-activated protein kinase (p38(AGF)) did not significantly affect apoptosis induced by PEITC. This result indicates that interfering with the JNK pathway prevents the PEITC-induced apoptosis, thereby suggesting that the JNK pathway is required for isothiocyanate-induced apoptosis.

Isothiocyanate-induced JNK Activation Is Inhibited by Antioxidants—JNK activity is regulated by the upstream kinase cascade (MEKK1 → MKK4 → JNK, 3); however, it is not clear which upstream signals regulate the JNK module after cells receive apoptotic stimuli. Isothiocyanates react with GSH and form dithiocarbamates (R–NH–C(=S)–SG) in the presence or absence of GST (43). Since apoptosis is activated by many oxidative agents (44), the isothiocyanates may induce oxidative

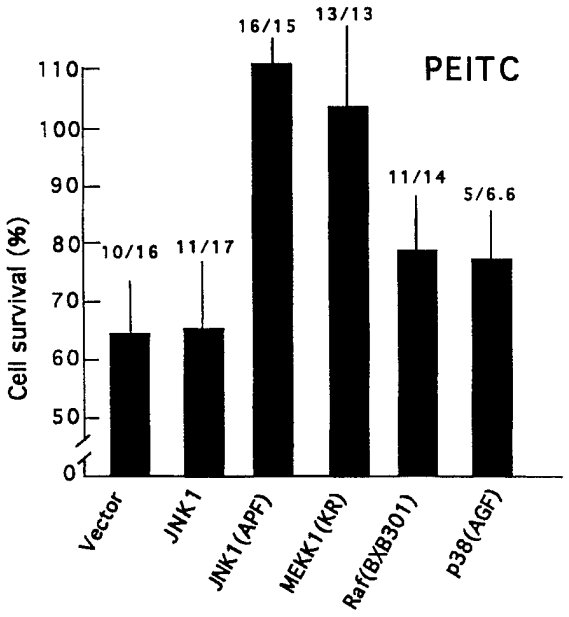

FIG. 5. **Suppression of PEITC-induced apoptosis by interfering with the JNK pathway.** 293 cells were transfected in duplicates with pCMV-βgal (1 μg) and mutant kinase-expressing vectors (3 μg for each) as indicated. Empty vectors were used to bring the total transfected DNA to 7 μg. After transfection, the cells were incubated in culture medium for 12 h and then treated with or without PEITC (20 μM). Cells were harvested 24 h after treatment, and the β-galactosidase-positive cells (blue color) were examined by enzymatic staining using X-gal as a substrate. Cell survival was determined as follows: (% of blue cells in treated group/% of blue cells in untreated group) × 100%. The data presented are the means ± S.D. of six experiments.

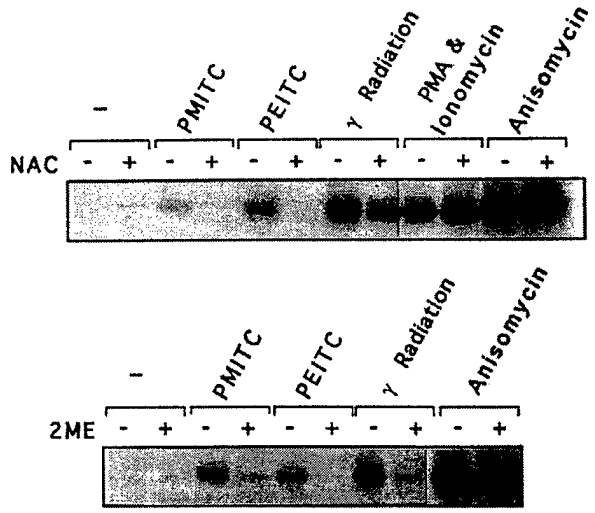

FIG. 6. **Inhibition of JNK activation by antioxidants.** Jurkat cells were pretreated with or without *N*-acetyl-L-cysteine (*NAC*; 20 mM) or 2-mercaptoethanol (*2ME*; 10 mM) and then treated with different stimuli: PMITC (5 μM, 2 h), PEITC (5 μM, 2 h), γ-radiation (100 gray, 2 h), phorbol 12-myristate 13-acetate (*PMA*; 50 ng/ml) plus ionomycin (1 μM), or anisomycin (2 μM, 30 min). JNK activity was assayed by immunocomplex kinase assays.

stress by reacting with and depleting the intracellular GSH pool, which may then induce JNK activation and apoptosis. We decided to test the roles of oxidation in isothiocyanate-induced JNK activation using antioxidants. JNK activation by PMITC and PEITC was inhibited by preincubation of the Jurkat cells with antioxidants, 2-mercaptoethanol (10 mM, 1 h) or *N*-acetylcysteine (20 mM, 2 h) (Fig. 6). The antioxidant treatments also blocked γ-radiation-induced JNK activation, but had no effect on JNK induction by phorbol 12-myristate 13-acetate plus ionomycin or by anisomycin (Fig. 6). The inhibition of JNK activation is not due to the loss of viability of Jurkat cells after antioxidant treatment because >95% of the cells retained the ability to exclude trypan blue 6 h after antioxidant treatment (data not shown). These data indicate that the JNK activation in cells exposed to PMITC, PEITC, or γ-radiation may be due to the induction of oxidative stresses. In contrast, the induction of JNK by phorbol 12-myristate 13-acetate plus ionomycin or by anisomycin may not be mediated through the intracellular oxidative changes.

Bcl-2 Suppresses PEITC-induced JNK Activation and Apoptosis—Bcl-2 family members are known to be important apoptosis regulators (45). We used a transient transfection/cell death protection assay to test if Bcl-2 or Bcl-x_L can block apoptosis induced by isothiocyanates. The levels of the Bcl-2 (Bcl-x_L) protein were examined by Western blot analysis (Fig. 7*A*), revealing the production of transfected genes. The empty vector control group lost 40% of cells after the drug treatment. In comparison, transfection of Bcl-2 or Bcl-x_L prevented PEITC-induced apoptosis in most of the transfected cells (Fig. 7*A*), indicating that Bcl-2 and Bcl-x_L were capable of protecting cells from PEITC-induced apoptosis. We also examined the PEITC-induced JNK activation in 293 cells transfected with empty vector or Bcl-2-encoding plasmids. Although only 40–50% of the cells were transfected, the endogenous JNK activity induced by PEITC was evidently decreased in Bcl-2-transfected cells (Fig. 7*B*). This result implicates Bcl-2 as an upstream suppressor for JNK activation by apoptotic stimuli.

To further examine the molecular ordering between Bcl-2 or Bcl-x_L and the JNK pathway, we cotransfected Bcl-2 or Bcl-x_L with JNK1 plus constitutively active MEKK1 (ΔMEKK1) into 293 cells. The transfection of JNK1 plus ΔMEKK1 led to persistent JNK activation and caused apoptosis independent of the upstream signals (Fig. 7*C*). Neither Bcl-2 nor Bcl-x_L significantly affected the cell death caused by JNK1 plus ΔMEKK1, although the levels of Bcl-2 and Bcl-x_L were increased in the transfected cells (Fig. 7*C*). In comparison to the Bcl-2- or Bcl-x_L-transfected cells, the lower levels of Bcl-2 and Bcl-x_L detected in the cells cotransfected with JNK1 plus ΔMEKK may be due to the loss of transfected cells caused by apoptosis (Fig. 7*C*). This result indicates that Bcl-2 and Bcl-x_L failed to prevent cell death induced by JNK activation; therefore, they may not be downstream of JNK in the apoptotic signaling pathway. Taken together, our results suggest that Bcl-2 and Bcl-x_L are upstream, but not downstream, of JNK in apoptotic signaling induced by isothiocyanates (Fig. 8).

DISCUSSION

Activation of the JNK pathway has been shown to be a common phenomenon in apoptotic cell death (24–27, 46); however, the importance of this activation seems to vary in apoptosis caused by different agents. The JNK pathway is required for apoptosis induction by growth factor withdrawal, heat shock, radiation, and ceramide (24–27). In contrast, JNK may not be essential for receptor-mediated apoptosis (*e.g.* Fas- and tumor necrosis factor-mediated apoptosis) (47, 48). In this study, we demonstrate the involvement of JNK in isothiocyanate-induced apoptosis by proving that interfering with the JNK pathway suppressed isothiocyanate-induced cell death. We observed that high doses of PMITC, PEITC, and PPITC (>50 μM) caused acute cell death in the absence of JNK activation; however, the cell death resembled necrosis rather than apoptosis. This result indicates that induction of JNK activity is not a general event caused by stress during cell death, but rather it is a specific phenomenon associated with apoptotic cell death. In addition, the failure of a caspase/ICE inhibitor to block JNK activation caused by isothiocyanates indicates that

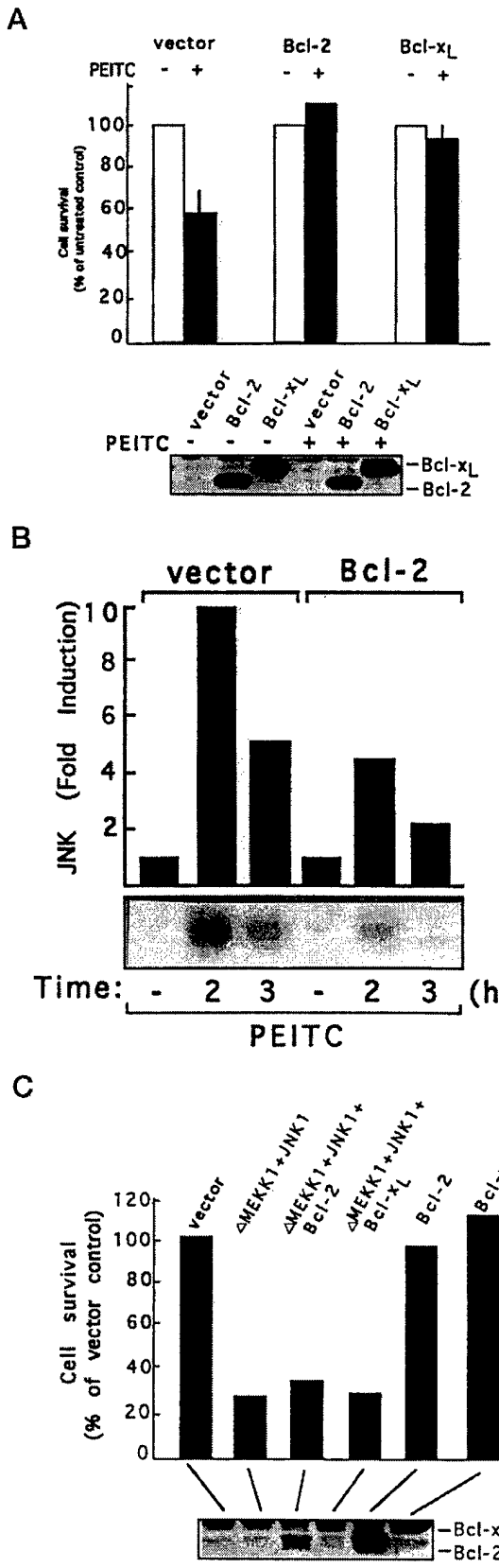

FIG. 7. **Suppression of PEITC-induced JNK activation and apoptosis by Bcl-2.** *A*, 293 cells were transfected with pCMV-βgal (1 μg) and control vector (3 μg) or Bcl-2- or Bcl-x_L-expressing plasmid (3 μg). Cells were cultured in complete medium for 12 h after transfection and then treated with or without PEITC (20 μM) for 24 h. Some of the harvested cells were assayed for cell survival as described under "Materials and Methods." The data presented are the means ± S.D. of three experiments. The remaining cells were lysed, and the expression of Bcl-2 and Bcl-x_L was determined by Western blot analysis. *B*, 293 cells were transfected with control vector or Bcl-2-encoding plasmid (3 μg), and transfected cells were treated for the indicated times with 20 μM PEITC 6 h after removing the transfection mixture. Endogenous JNK activity was examined by immunocomplex kinase assays. *C*, 293 cells were transfected with pCMV-βgal (1 μg) plus different combinations of plasmids as indicated (JNK1, 1.5 μg; ΔMEKK1, 1.5 μg; and Bcl-2 or Bcl-x_L, 3 μg). Empty vector was added to normalize the total DNA amount. Cells were collected 48 h after transfection, fixed, stained, and examined as described under "Materials and Methods." The cell survival data presented are the means of three experiments. Western blotting for Bcl-2 and Bcl-x_L was performed as described under "Materials and Methods."

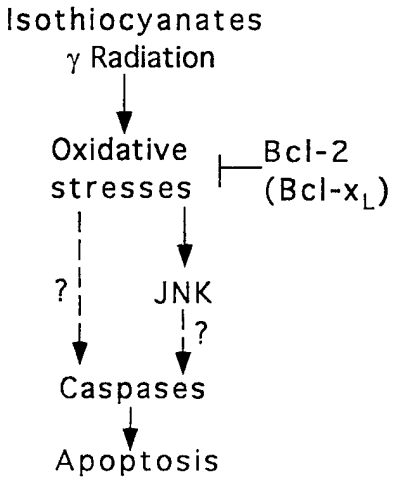

FIG. 8. **Model of molecular mechanisms of JNK-mediated apoptotic signaling.**

JNK activation may initiate apoptotic signaling and that it is not a secondary effect of cellular damage from apoptotic cell death. However, we (26) and others (48) have shown that JNK activation in Fas-mediated apoptosis can be suppressed by a caspase/ICE inhibitor, which suggests that JNK induction could be augmented by activation of caspases/ICE-like proteases. A recent report indicates that CPP32, a caspase/ICE-like protease, is capable of cleaving D4-GDI, a GDP dissociation inhibitor of the Ras-related Rho family GTPase (49). The process of D4-GDI may irreversibly activate the Rho family G proteins, which are activators of the JNK pathway (50, 51). Therefore, the activation of CPP32 may lead to the enhancement of JNK activation. If this regulatory mechanism exists, it may work as a signaling circuit to amplify the apoptotic signal, but it may not be essential for Fas-mediated apoptosis.

Our result indicates that oxidative stress may initiate JNK activation (Fig. 6). The anti-apoptotic regulator Bcl-2 may inhibit apoptosis by suppressing the formation or the damaging effects of reactive oxygen species (ROS) (52, 53), which are generated by many apoptotic agents (44). In this report, we showed that Bcl-2 suppressed PEITC-induced JNK activation. Bcl-2 or Bcl-x_L blocked apoptosis caused by PEITC, but failed to suppress apoptosis caused by overexpression of activated JNK1. In addition, a recent report showed that Bcl-2 blocks JNK activation induced by serum depletion or nerve growth factor withdrawal in PC-12 cells (54). Taken together, Bcl-2 may be an upstream suppressor of the JNK pathway acting to relieve the oxidative stress and to prevent JNK induction and the initiation of apoptotic signals (Fig. 8). The failure of Bcl-2 or Bcl-x_L to block cell death induced by JNK1 plus ΔMEKK1 suggests that Bcl-2 may not be downstream of JNK. However, these data do not exclude the possibility that JNK may directly or indirectly down-regulate the function of Bcl-2, therefore causing cell death. The tumor suppressor p53, which is essen-

tial for ionizing radiation-induced apoptosis (55), has been shown to be a substrate of JNK (56). p53 is a positive regulator for the expression of Bax (57), a counteracting molecule of Bcl-2 and a potent apoptosis inducer (58). Although the exact physiological function of this p53 phosphorylation is still unclear, p53 may be a mediator for JNK-induced apoptosis.

Isothiocyanates are well known for their chemopreventive effects on various carcinogens (29–33). Previous studies attributed this anticarcinogenic property to their ability to affect the bioactivation, detoxification, and excretion of carcinogens (34–37). Here, we show that isothiocyanates induced apoptosis by activation of the JNK pathway. We propose that isothiocyanate-mediated apoptosis may be one possible mechanism to achieve the anticarcinogenic function. Carcinogens usually cause genomic damages in the exposed cells. If the damages are limited, the cells can repair those damages and maintain normal functions. The cells that fail to repair the damages are normally eliminated by apoptosis, which prevents the propagation of genomic damages to progenitor cells. The cells with genomic damages that escape cell death are prone to develop into cancerous cells. In the presence of isothiocyanates, the cells will start the apoptosis process because of an increase in oxidative stress and JNK activity. The simultaneous or subsequent exposure to a carcinogen will trigger the cell death progression, and the damaged cells will be eliminated by apoptosis; therefore, fewer cells can survive and become cancer cells. In addition, cancer cells usually have a higher metabolic rate and generate higher levels of intracellular oxidants than normal cells. The ability of isothiocyanates alone to generate oxidative stress, activate the JNK pathway, and induce apoptosis suggests that they may have a therapeutic function in addition to their chemopreventive functions.

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